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14. ABSTRACT

The goal of this study is to evaluate if promotion of angiogenesis by systemic treatment with an antagomir against miR-92a, a well established inhibitor of angiogenesis, will maximize the benefits of exercise on bone. 10 week old female C57BL6/J mice were subjected to two weeks of external load by four point bending. During the first week of mechanical loading (ML), mice were injected (2.7 mg/kg of bodyweight) with antagomir against miR-92 or control antagomir (3 alternate days via retro-orbital). No difference in tissues weights (heart, kidney, liver) were found in mice treated with miR-92 vs. control antagomir suggesting no side effects. 2 weeks of ML increased tibia TV, BV/TV and density by 6-15%, as expected, in the control antagomir treated mice. Similar increases in the above parameters (7-16%) were also seen in mice treated miR-92 antagomir. Administration of miR-92 antagomir was effective in reducing levels of mir-92 in heart, liver and skeletal muscle and in contrast, expression levels of two other MicroRNA's miR-93 and miR-20a remain constant, thus suggesting specificity of the antagomir used. Surprisingly, we failed to detect significant changes in the expression levels of vascular genes (VEGF, CD31 and Tie2) in heart, liver or skeletal muscle. Based on these findings, we conclude that systemic administration of antagomir against miR-92 while reduced expression levels of miR-92 in the tissues; it did not significantly alter either angiogenic or osteogenic response, thus suggesting possible redundancy in miR-92 regulation of angiogenesis.

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Introduction

Bone is a highly vascularized tissue that is reliant on the close spatial and temporal connection between blood vessels and bone cells to maintain skeletal integrity. Several experimental studies have shown that angiogenesis plays a vital role in skeletal development, repairing fractured bone and in response to mechanical loading (ML). These include: 1) Studies using a distraction osteogenesis model (DO) have shown that intramembranous bone formation is induced by the application of gradual mechanical distraction across an osteotomy defect, which reveals not only an increase in osteogenesis but also an increase in expression of several angiogenic factors (1). 2) Treadmill-running in rats displayed bone marrow angiogenesis concomitant with increase in osteogenesis (2). 3) Studies using mandibular DO model have shown that high frequency traction provides a proper mechanical environment for angiogenesis contributing to enhanced bone formation (3). These findings illustrate that angiogenesis, osteogenesis and ML are tightly associated.

Recently, newly discovered MicroRNA's (miR) belonging to a small class of RNA molecules have received considerable attention because of the ability to act as negative regulators of gene expression. RNA of this type regulates gene expression at the post-transcriptional level by either degradation or translational repression of a target mRNA. So far, at least 500 miR have been discovered of which few are linked to pathogenesis of disease (heart disease, cancer and schizophrenia) as evident from human and animal studies (4-6). In particular, reports have shown that microRNA regulates angiogenesis. Since angiogenesis and osteogenesis is tightly coupled, we predict that inhibiting microRNA that control angiogenesis can maximize the benefits of exercise on skeleton.

Our second aim in the proposed grant was to test if blocking microRNA regulating angiogenesis increases the benefits of exercise on skeleton.

Specific aim 2: Determine the consequence of blocking identified MicroRNA using antisense oligos complementary to the specific MicroRNA that control angiogenesis on loading induced bone formation.

1) Using an *in vitro* model, determine the efficiency of antagomirs specific for identified MicroRNA that control angiogenesis.
2) Based on *in vitro* results and specific aim 1, we will inject mice with antisense oligos (antagomirs) specific for MicroRNA that control angiogenesis.
3) Subject this experimental group and control mice to axial loading for 2 weeks.
4) Measure ML induced changes in skeletal parameters and bone strength by Micro-CT.
5) Measure cellular processes contributing to osteogenesis and angiogenesis by performing histology.

Findings

During the first year of the funding, using expression profile, we have identified microRNAs that are associated with angiogenesis and osteogenesis. Although many were identified, the issue of which microRNA having a high negative effect on osteogenesis and angiogenesis needs further evaluation. Since testing the biological effect of each identified miR with specific antagomir is expensive, we focused on the miR-92 (7) for several reasons, which include 1) miR-92 has been reported to be a negative regulator of angiogenesis. 2) Over expression of miR-92 blocked angiogenesis in human endothelial cells and that blocking miR-92 recovered mouse from limb ischemia injury as well as from myocardial infarction. Therefore, based on these observations, we

predicted that inhibiting MicroRNA-92 that control angiogenesis can maximize the benefits of exercise on skeleton.

Animals: To test the above prediction, 9 week old female C57BL/6J mice were purchased from Jackson Laboratory. All the mice were housed under the standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The experimental protocols were in compliance with animal welfare regulations and approved by local IACUC.

Antagomir designing: To block miR-92, a microRNA that is well known in regulating angiogenesis, single stranded RNA antagomir sequences against miR-92 and control antagomir sequence were ordered from IDT DNA technology. The sequences were obtained from previously published manuscript (7). In the miR-92 antagomir and control antagomir, the 2'O RNA base was methylated followed by first two bases and last 3 bases were phosphorothiated to increase the stability of antagomir from degradation. In addition, a cholestrol-TEG was added at the 3' for easy entry of antagomir into the cells. The sequence of antagomir for miR-92a is as follows: 5`-CAGGCCGGGACAAGUGCAAUA-3`) and Antagomir-Co (5`- AAGGCAAGCUGACCCUGAAGUU-3`.

Four-point bending in antagomir treated mice and micro-CT measurement of skeletal parameters: To test, if blocking miR-92 maximizes the benefit of exercise on bone, we performed ML using four-point bending method on 10 week old female B6 mice for a period of 2 weeks (8-10). We choose this model and the load based on our findings that showed high osteogenic and angiogenic effect in response to loading during the first year of funding. We applied a 9N load on the right tibia of B6 mice over the muscle and soft tissue at 2Hz frequency, 36 cycles once per day under inhalable anesthesia (5% Isoflurane and 95% oxygen) for a period of 2 weeks (6 days/week with 1 day rest). The left tibia was used as contra-lateral internal control.

Several studies have used antagomir to block target microRNA at a concentration ranging from 0.33 mg to 100 mg/kg of bodyweight in animal models. The issue of whether the doses used are optimal and specific to target microRNA has not been examined thoroughly. However, one potential concern with the use of high dose of antagomir is that antagomir at high concentrations could produce non-specific effects by inhibiting other genes besides target gene. Since reports have shown 0.33- 1.0- and 3.3- mg/kg of dosage was effective in exhibiting the biological response in tissues (11), we chose a dose of 2.7mg/kg of bodyweight antagomir for our study. To determine whether blocking miR-92 will maximize the benefits of exercise on bone via increasing angiogenesis, we injected antagomir against miR-92 and control antagomir via retro-orbital approach under 5% Isoflurane and 95% oxygen anesthesia for a period of one week (3 alternate days) while the mice were subjected to loading regimens. After two weeks of loading, we measured mice body weight and tissues weights after euthanization followed by tibias were collected to evaluate if there is an increase or decrease in loading induced changes in skeletal parameters by using micro-CT (Scanco Invivo CT40, Switzerland) and histology (10).

After two weeks of loading, we found no significant difference in the body weight between control and experiment group (Table-1). Similarly, we did not see any difference in the tissue weights between the control and experimental groups suggesting that there are no side effects caused by the antagomir injections (Table-2).

Table -1 Body weight and tissue weight measurements in mice treated with antagomir (experimental group) vs. control antagomir (control group) after two weeks of four-point bending in 10 week old female B6 mice.

Tissues	Control group (grams)	Experimental group (grams)
Body weight	19.6 ± 0.1	19.7 ± 0.26
Kidney	0.10 ± 0.006	0.098 ± 0.005
Liver	0.88 ± 0.02	0.83 ± 0.04
Heart	0.10 ± 0.006	0.11 ± 0.010

N=4, Values are mean ± SD.

Micro-CT analysis revealed that two weeks of ML increased tibia tissue volume (TV), bone volume/tissue volume (BV/TV) and bone density by 6-15%, as expected, in the control antagomir treated mice. Surprisingly, similar increases in TV (16%), BV/TV (9%) and bone density (7%) were also seen in mice treated with miR-92 antagomir (**Figure 1**). Since the antagomir used did not sensitize the skeleton to mechanical loading, as we predicted, we did not perform any histomorphometric analysis on the bone samples from this study.

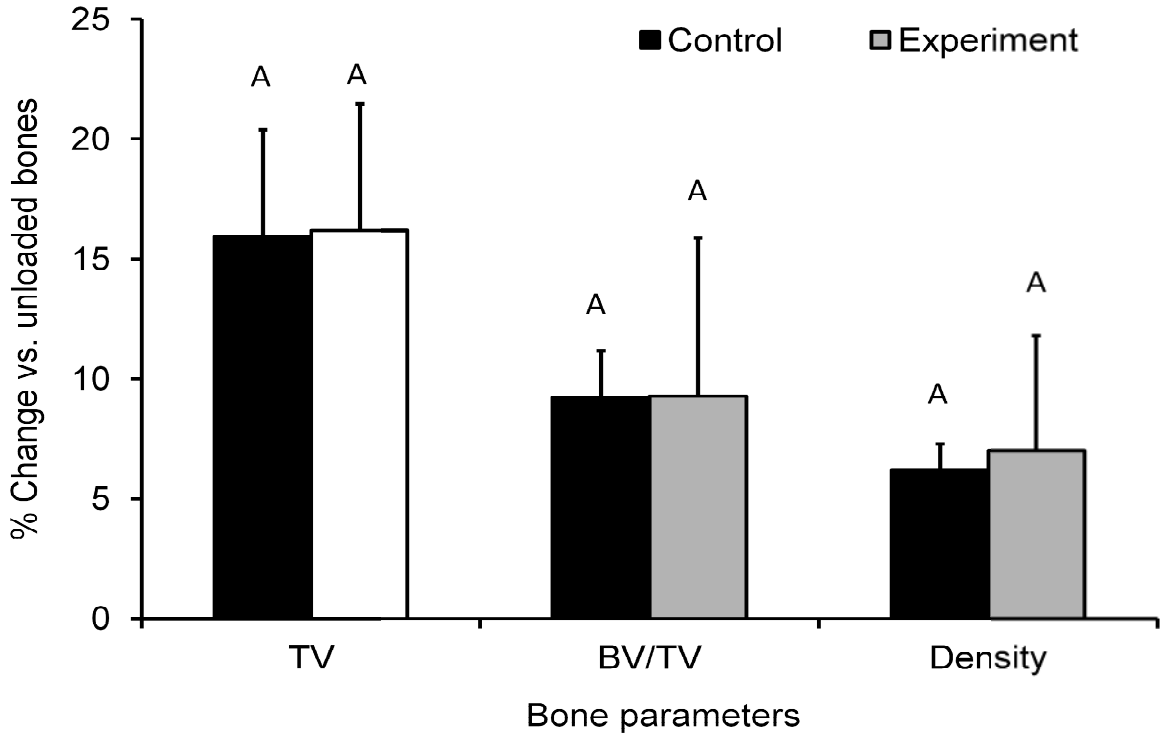

Figure 1 – Micro-CT measurements of bone parameters (diaphysis area) after 2 weeks of loading in female B6 mice treated with control antagomir and antagomir against miR-92. The y-axis corresponds to percent change and x-axis represents bone parameters. TV: Tissue volume, BV/TV: Bone volume/Tissue volume and density: total bone density. Values are mean ± SD, N=4. $^{A}p<0.05$ vs. unloaded bones.

Since there was no difference between the groups, this, then raises a question whether systemic administration of mir-92 antagomir was effective in reducing the levels of miR-92. To test this, RNA was extracted from liver, heart, and skeletal muscle of antagomir and control antagomir treated mice using Trizol followed by Direct-zol kit (Zymo Research, USA). Quality and quantity of RNA were analyzed using a 2100 Bio-analyzer (Agilent, Palo Alto, CA, USA) and Nano-drop (Wilmington, DE). To assure that the antagomir delivered into mouse model blocked miR-92, expression levels of miR-92, U6 (internal control) and two other MicroRNA's (miR-93 and miR-20a) to determine the specificity were quantitated in liver, skeletal muscle and heart of mir-92 antagomir and control antagomir treated mice. Levels of MicroRNA's were evaluated using microRNA specific stem-loop RT primer and by Real time PCR primer that were obtained from Applied Biosystem and reactions were performed according to the manufacture instructions. In brief, 10ng of RNA was used synthesize the first strand of cDNA by reverse transcription using respective stem-loop microRNA RT primer. Approximately 10µl of reaction volume was used for the RT assay that consisted of 0.1µl of 100 dNTPs, 1µl of 10X Buffer, 0.5µl of reverse transcriptase enzyme, 0.12µl of 50U/µl RNasin, 1µl of 5X RT primer, 6.78µl of Nuclease free water and 0.5µl of 10ng RNA. 5µl of the five times diluted first strand cDNA reaction was subjected to real time PCR amplification using

microRNA specific real time PCR primers. Approximately 20µl of reaction volume was used for the assay that consisted of 10µl of TaqMan 2X Universal PCR Master mix, no AmpErase UNG, 0.5µl of 20X probe against target microRNA and 4.5µl of Nuclease free water, and 5µl of DNA. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using U6 as internal control and the normalized values were subjected to a $2^{-\Delta\Delta}$Ct formula to calculate the fold change between the antagomir and control antagomir treated groups. The results from our study revealed a 5-14 fold decrease in miR-92 levels in the heart, liver and skeletal muscles of mice treated with miR-92

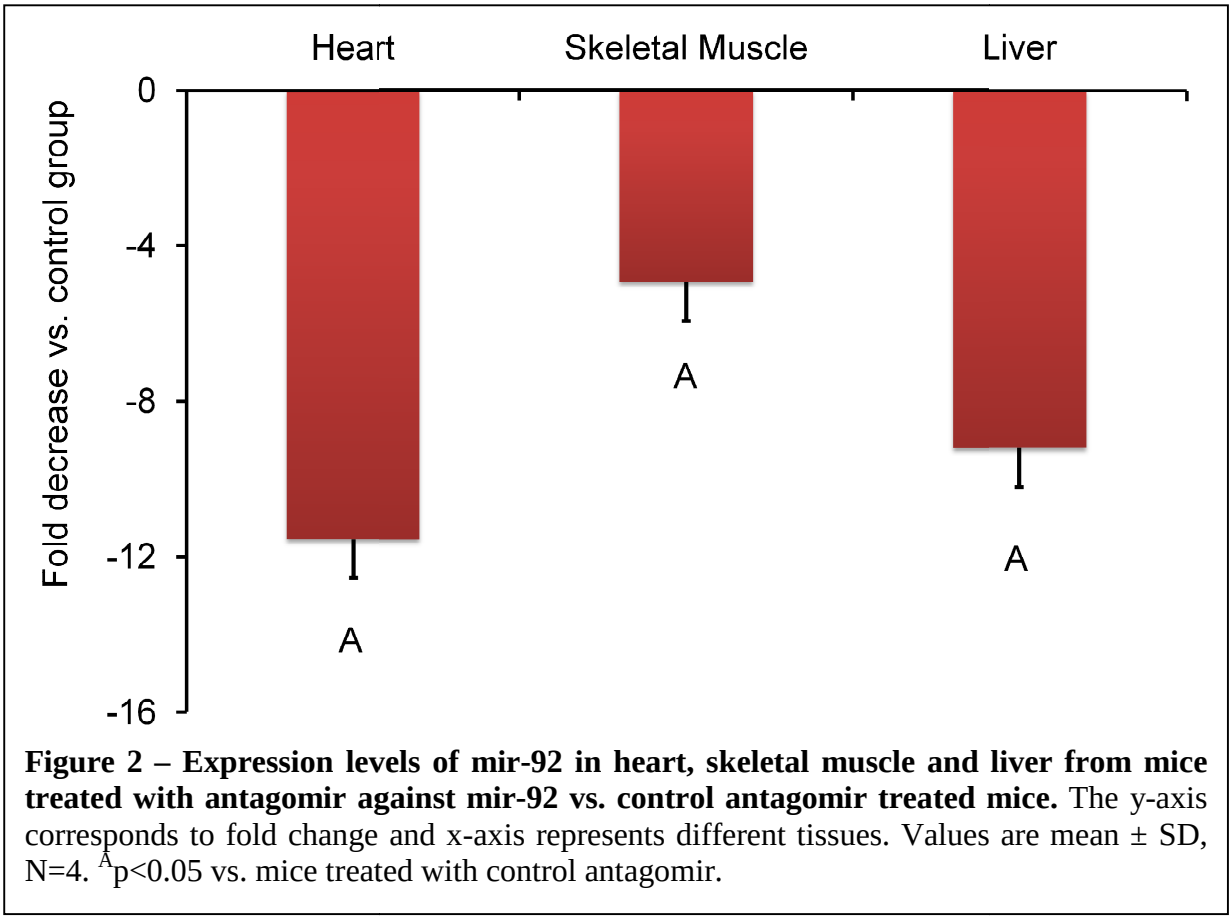

Figure 2 – Expression levels of mir-92 in heart, skeletal muscle and liver from mice treated with antagomir against mir-92 vs. control antagomir treated mice. The y-axis corresponds to fold change and x-axis represents different tissues. Values are mean ± SD, N=4. $^{A}p<0.05$ vs. mice treated with control antagomir.

antagomir compared to control antagomir (**Figure 2**). In contrast, expression levels of two other MicroRNA's miR-93 (fold change vs. control antagomir = 1.0 ± 0.6) and -20a (0.88 ± 0.18) were not different between the two groups of mice, thus suggesting specificity of the antagomir used. Since past reports have shown that mir-92 is an important regulator of angiogenesis, we also determined whether blocking miR-92 maximized the expression levels of vascular marker genes in the above tissues samples. We used CD31, Tie2 and VEGF genes to evaluate angiogenesis since reports have shown that these two genes are highly expressed on the surface of the endothelial cells at sites of neo-vascularization or vascularization and an increase in the expression levels of these genes corresponds to an increase in vascularization (12-14). To quantitate expression levels of vascular genes, briefly, purified total RNA [200µg/µl] was used to synthesize the first strand cDNA by reverse transcription according to the manufacturer's instructions [Bio-Rad, Hercules, CA USA]. 5µl of the five times diluted first strand cDNA reaction, was subjected to real time PCR amplification using gene specific primers as described earlier (9, 10). The gene specific primers for angiogenesis (CD 31, Tie 2) were designed by using Vector NTI software and ordered from IDT DNA technologies (USA). Approximately 20µl of reaction volume was used for the real time PCR

assay that consisted of 1X [10µl] Universal SYBR green PCR master mix [Master mix consists of SYBR Green dye, reaction buffers, dNTPs mix, and Hot Start Taq polymerase] [Applied Biosystems, Foster City, CA], 50nM of primers, 15µl of water, and 5µl of template. The thermal conditions consisted of an initial denaturation at 95ºC for 10 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds (sec), annealing and extension at 60ºC for 1 minute, and a final step melting curve of 95ºC for 15 sec, 60ºC for 15 sec, and 95ºC for 15 sec. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using the endogenous control [β-actin] and the normalized values were subjected to a $2^{-\Delta\Delta}$Ct formula to calculate the fold change between the antagomir and control antagomir treated mice.

Surprisingly, we failed to detect any significant changes in the expression levels of vascular genes (VEGF, CD31 and Tie2) in heart, liver or skeletal muscle at the time points examined (**Figure 3**). We offer the following potential explanations for the negative results in this study: 1) the dosage of antagomir used in our study was slightly less compared to earlier study (2.7 mg/kg of body weight vs. 8 mg/kg of body weight) (7). The issue of whether higher dosage is required to see a change in the phenotype or whether the inhibitory effect on angiogenesis seen in the previous study was due to secondary effects on other genes require further evaluation. 2) The expression levels of angiogenic genes were examined only at one time point and additional time points may need to be examined. 3) Earlier study measured an increase in angiogenesis by blood flow while we examined expression levels of vascular genes.

The data presented above are given as mean ± SD. Standard t-test was used to compare the difference between externally loaded versus non-loaded bones. We used Statistica software (StatSoft, Inc version 7.1, 2005) to perform the analysis and the results were considered significant at $p<0.05$.

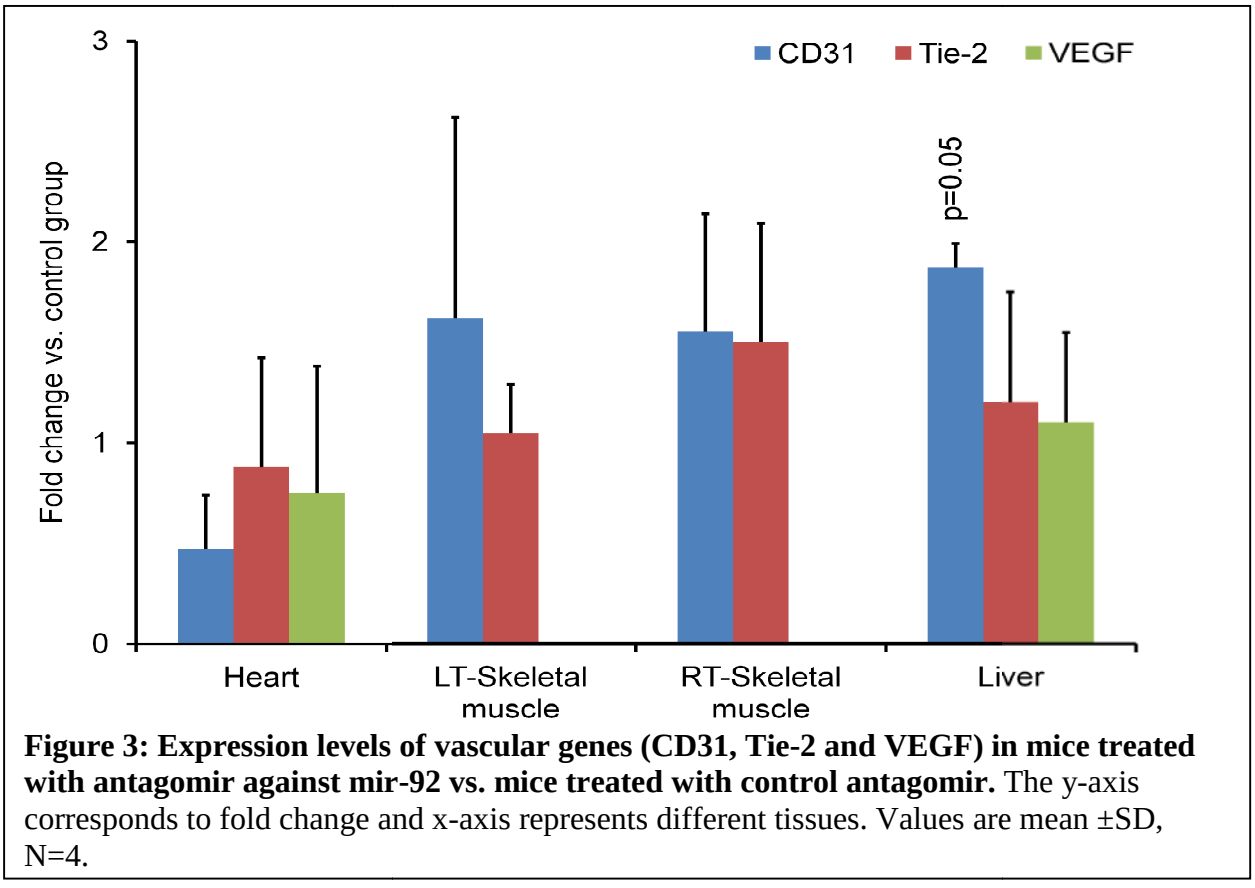

Figure 3: Expression levels of vascular genes (CD31, Tie-2 and VEGF) in mice treated with antagomir against mir-92 vs. mice treated with control antagomir. The y-axis corresponds to fold change and x-axis represents different tissues. Values are mean ±SD, N=4.

Conclusion

Based on our above findings, we conclude that systemic administration of antagomir against miR-92, while it reduced expression levels of miR-92 in the skeletal muscle, liver and heart; it did not significantly alter either angiogenic or osteogenic response, thus suggesting possible redundancy in miR-92 regulation of angiogenesis.

Current Progress

Since there is a discrepancy between our findings and the published report, we focused on generating bone specific microRNA knockout mice to test the role of the microRNAs involved in regulating angiogenesis and osteogenesis in relation to exercise.

Reportable Outcome

1. Anthony S, Santisuk R, Xing W, Kesavan C. Systemic administration of an antagomir designed to inhibit miR-92, a regulator of angiogenesis, failed to modulate skeletal anabolic response to mechanical loading. *Physiol Res*. 2012 Dec 13.

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